Cell-to-cell movement of *Alfalfa mosaic virus* can be mediated by the movement proteins of Ilar-, bromo-, cucumo-, tobamo- and comoviruses and does not require virion formation

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Received 9 June 2005; returned to author for revision 29 June 2005; accepted 7 October 2005

Available online 28 November 2005

**Abstract**

RNA 3 of *Alfalfa mosaic virus* (AMV) encodes the movement protein (MP) and coat protein (CP). Chimeric RNA 3 with the AMV MP gene replaced by the corresponding MP gene of *Prunus necrotic ringspot virus*, *Brome mosaic virus*, *Cucumber mosaic virus* or *Cowpea mosaic virus* efficiently moved from cell-to-cell only when the expressed MP was extended at its C-terminus with the C-terminal 44 amino acids of AMV MP. MP of *Tobacco mosaic virus* supported the movement of the chimeric RNA 3 whether or not the MP was extended with the C-terminal AMV MP sequence. The replacement of the CP gene in RNA 3 by a mutant gene encoding a CP defective in virion formation did not affect cell-to-cell transport of the chimera’s with a functional MP. A GST pull-down technique was used to demonstrate for the first time that the C-terminal 44 amino acids of the MP of a virus belonging to the family Bromoviridae interact specifically with AMV virus particles. Together, these results demonstrate that AMV RNA 3 can be transported from cell-to-cell by both tubule-forming and non-tubule-forming MPs if a specific MP–CP interaction occurs.

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**Keywords:** Alfalfa mosaic virus; Movement proteins; Cell-to-cell movement; GFP; Ilarvirus; Bromovirus; Cucumovirus; Tobamovirus; Comovirus

**Introduction**

After the establishment of an infection site in a compatible host, a plant virus moves from cell-to-cell to reach the vascular system. Entry into the vascular system then permits long-distance transport of the virus from the infected leaf to distal parts of the plant (Carrington et al., 1996; Lazarowitz and Beachy, 1999; Waigmann et al., 2004). Cell-to-cell movement of the virus occurs via plasmodesmata and involves one or more movement proteins (MPs) encoded by the viral genome. For plant viruses with an RNA genome, two main mechanisms of cell-to-cell movement have been characterized, which are exemplified by *Tobacco mosaic virus* (TMV, genus *Tobamovirus*) and *Cowpea mosaic virus* (CPMV, genus *Comovirus*). TMV MP alters the size exclusion limit of plasmodesmata and mediates cell-to-cell transport of a complex of viral RNA and MP together with the viral replication complexes by a mechanism that does not involve the viral coat protein (CP) (Citovsky et al., 1992; Heinlein et al., 1995; Kawakami et al., 2004; McLean et al., 1995; Wolf et al., 1989). On the other hand, CPMV MP forms tubular structures which traverse plasmodesmata and guide the transport of virions to a neighboring cell (Kasteel et al., 1993; van Lent et al., 1990; Wellink and Vankammen, 1989). In spite of the different transport mechanisms, both MPs have been assigned to the 30K superfamily of viral MPs that are structurally related to the 30 kDa MP of TMV (Melcher, 2000).

In the family Bromoviridae, studies on cell-to-cell movement have been mainly focused on *Alfalfa mosaic virus* (AMV, genus *Alfamovirus*), *Brome mosaic virus* (BMV, genus *Bromovirus*), *Cucumber mosaic virus* (CMV, genus *Cucumovirus*) and *Prunus necrotic ringspot virus* (PNRSV, genus *Ilarvirus*). Available data support the notion that BMV moves cell-to-cell in the form of virions (Okinaka et al., 2001; Schmitz and Rao, 1996), while CMV moves as a ribonucleoprotein complex (Kaplan et al., 1998). The transport mechanism of AMV has been reported to share characteristics with those of both TMV and CPMV (Kasteel et al., 1997; Sanchez-Navarro and Bol, 2001). Here, we have analyzed the cell-to-cell movement of AMV in further detail and demonstrated that AMV RNA 3 can...
be transported by tubule-forming and non-tubule-forming MPs. The AMV genome consists of three plus-strand RNAs. Monocistronic RNAs 1 and 2 encode the P1 and P2 polymerase proteins. The dicistronic RNA 3 encodes the MP and CP; expression of the CP gene occurs via a subgenomic messenger RNA 4. Both MP and CP are required for virus transport. The C-terminal 44 residues of the MP of AMV are dispensable for tubule formation, cell-to-cell and systemic transport of the virus (Sanchez-Navarro and Bol, 2001). It has been postulated that the C-termini of the MPs of AMV, BMV, CMV and Tomato aspermy virus (TAV, genus Cucumovirus) are involved in an interaction with the cognate viral CP (Nagano et al., 1999; Salanki et al., 2004; Sanchez-Navarro and Bol, 2001; Takeda et al., 2004), but as far as we know, this interaction has not been demonstrated so far. In the present study, we have replaced the MP gene of AMV by the MP genes of BMV, PNRSV, CMV, TMV or CPMV with or without an extension encoding the C-terminal 44 residues of AMV MP. Cell-to-cell movement of the chimeric RNA 3 molecules was studied in transgenic tobacco plants expressing the AMV P1 and P2 proteins (P12 plants). In addition, we present in vitro results that demonstrate the capability of the C-terminal region of the AMV MP to interact specifically with purified AMV virus particles. Finally, the role of virion formation in cell-to-cell transport was studied.

Results and discussion

Transcripts made with T7 RNA polymerase from the AMV RNA 3 chimeric constructs carrying the MP gene of TMV, CPMV, PNRSV, BMV and CMV were inoculated on P12 plants. Fig. 1 shows the fluorescent signal monitored by confocal laser scanning microscopy (CLSM) 2 days post-inoculation (dpi). All RNA 3 hybrids carrying the extended C-terminal 44 aa of AMV MP showed clear infection foci (compare left and right panels, Fig. 1). Only AMV and TMV MPs were able to transport the viral genome in absence of the AMV MP C-terminal extension (see left part of Fig. 1A). Consistently with this result, it was previously shown that the MP of a Bromovirus (Cowpea chlorotic mottle virus) can replace the corresponding TMV MP for systemic infection (DeJong and Ahlquist, 1992). Similar results were observed at 4, 6 and 10 dpi (data not shown). RNA accumulation levels of the different hybrids were analyzed in P12 protoplasts as described previously (Sanchez-Navarro et al., 2001). The presence or absence of the C-termini of the AMV MP did not affect RNA accumulation (compare Figs. 2A and B). Chimeric RNA 3 and 4 accumulated to comparable levels to the unmodified GFP AMV RNA 3 (70.5 to 100%). However, the new generated subgenomic RNA (sgRNA) accumulated at lower levels than the corresponding unmodified GFP AMV RNA 3. Since the construct given the lowest accumulation level for the sgRNA (TMV 268; Figs. 2A, B: lane 5) was able to move cell-to-cell, the absence of foci in the rest of chimeras cannot be attributed to the low levels of the corresponding sgRNA. In addition, it has been reported that AMV mutants that accumulated at 5% of the level of the wild type in protoplasts were able to induce infection foci of wild-type size (Sanchez-Navarro et al., 2001; Tenllado and Bol, 2000). From the previous results obtained with the MPs of AMV, BMV and CMV, it was postulated that their C-termini specifically interact with their cognate CPs but not with heterologous CPs (Nagano et al., 1999; Sanchez-Navarro and Bol, 2001; Takeda et al., 2004). Thus, the added C-terminal AMV MP sequence allows the BMV, CMV and CPMV MPs to interact with AMV CP in the process of cell-to-cell transport. The capacity of the MP of AMV to interact with the cognate CP was investigated by GST pull-down technique. Different GST-MP fusions (Fig. 3A) were overexpressed and purified by using glutathione-sepharose 4B beads. The resultant GST-MP-bead complex was purified and incubated with AMV virus particles. After the incubation and washes steps, the proteins bound to the GST-MP beads were analyzed by Western blot using an antibody against the CP of AMV (Fig. 3B). As expected, CP was found to associate to full-length AMV MP but not with PNRSV MP (Fig. 3B, lanes 1 and 2), indicating the capacity of the CP of AMV to interact specifically with the cognate MP. Similar specific CP interaction was observed when the C-terminal 47 amino acids of the AMV MP were fused to the GST either alone or fused to the C-terminal 48 amino acids of the BMV MP (Fig. 3B, lanes 3 and 5, respectively). Negative CP signal was observed with either the C-terminal region of the BMV MP and the free GST (Fig. 3B, lanes 4 and 7, respectively). The observation that the C-terminal region of the AMV MP retains the capacity to interact with AMV virus particles in heterologous proteins in vitro strongly supports the previous hypothesis by which the functional MPs extended with the C-terminal 44 amino acids of the AMV MP should interact with the AMV CP in vivo. In the case of the CPMV MP, the C-terminal 48 amino acids have been shown to bind specifically to CPMV virions, interacting to the large CP subunit (Carvalho et al., 2003). Possibly, the inability of the CPMV MP to support the AMV transport (Fig. 1A, CPMV MP) reflects an incompatible interaction between the C-terminus of the MP protein and the CP of AMV. Because CPMV:A44 is functional in AMV cell-to-cell transport, the virion-binding domain of the MP of CPMV probably does not interfere with the binding of the C-terminal AMV-sequence of the chimeric MP to AMV CP (Fig. 1A, CPMV MP:A44). For TMV MP, the C-terminal extension was not required to allow the virus movement, indicating that the transport of AMV RNA by this MP is very probably independent of CP. Similar CP-independency was observed with the truncated MP of BMV and CMV lacking the C-termini (Nagano et al., 1997; Sanchez-Navarro and Bol, 2001), strongly suggesting that this part of the MP is required for the recognition of the cognate CP. The question remains then why some MPs (e.g., TMV) or truncated MPs (e.g., CMV and BMV MPs) do not require the cognate CP for the virus movement, meanwhile, other ones do. In the case of the truncated CMV MP, lacking the C-terminal region required for the putative CP interaction, the RNA affinity has been shown to be higher than the CP-dependent full-length MP (Andreev et al., 2004; Kim et al., 2004). This observation opens the possibility that the C-terminal region of the CMV MP could modulate, via a CP interaction, the RNA binding properties of the MP by a change of the protein conformation or perhaps by
the incorporation of the CP RNA binding capacity. A similar
function could explain the positive effect of the C-terminus of
the MP of AMV in the hybrid proteins described herein. The
idea of an MP with different RNA binding affinities modulated
by its interaction (high affinity) or no (low affinity) with the CP
is very attractive to explain the behavior of different MPs. Thus,
the RNA affinity of the CP-dependent MPs should be insufficient to allow virus transport, meanwhile, it should be enough for the CP-independent ones. In this sense, we have shown that small variations of the RNA binding affinity of the PNRSV MP makes this protein unable to allow the virus transport (Herranz et al., 2005).

To know whether or not the position of the C-terminal AMV MP sequence was important in allowing the MP–CP recognition and thus facilitating the cell-to-cell movement, the MP gene of AMV RNA 3 was replaced by the chimeric BMV255:A44:B48 gene. In BMV255:A44:B48, the C-terminal 44 amino acids of the AMV MP were introduced in the MP of BMV before its C-terminal 48 amino acids. This C-terminal region of the MP of BMV has been reported to be dispensable for tubule formation and cell-to-cell transport (Sanchez-Navarro and Bol, 2001). As can be observed in Fig. 1B, BMV255:A44:B48 is as active as BMV303:A44 in cell-to-cell transport of AMV. Thus, the C-terminal 44 amino acids of AMV MP are required to facilitate the cell-to-cell movement of the AMV/BMV chimera regardless of its position within BMV MP.

Both TMV and CPMV MPs are functional in the RNA 3 of AMV, suggesting the capacity of the virus to use the two different transport mechanisms: by the formation of a ribonucleoprotein complex and by a virion tubule-guide mechanism. This is the first time that both MPs have been shown to be interchangeable in the same viral system. In spite of the differences in transport mechanisms, both MPs belong to the '30K superfamily' whose components have been proposed to have a common predicted core structure flanked by variable N- and C-terminal domains (Melcher, 2000). The observation of a functional complementation between movement functions of tobamo- and comoviruses (Taliansky et al., 1993) together with the results presented in this work support a common functionality for the all MPs of the family. However, we cannot exclude the possibility that each MP, in the AMV RNA 3 context, was transporting their expected viral entity: e.g., the ribonucleoprotein intermediates with the MP of TMV and the virus particles with the MP of CPMV.

The chimera AMV RNA 3 constructs carrying the hybrid MPs BMV:A44, CPMV:A44, TMV:A44 and TMV were modified to express a CP mutant, lacking the C-terminal 21 amino acids (mutant CP-N199), which is defective in virion assembly but competent to accumulate substantial levels of plus RNA (compare Figs. 2A and 2C, lane 1) and to cell-to-cell spread (Sanchez-Navarro and Bol, 2001; Tenllado and Bol, 2000). Except for the sgRNA, all constructs accumulated comparable levels of the chimeric RNA 3 and RNA 4 on P12

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**Fig. 1.** Detection of infection foci in P12 plants after inoculation with a hybrid *Alfalfa mosaic virus* (AMV) in which its movement protein (MP) gene was exchanged by different movement protein genes. (A) P12 plants were inoculated with RNA transcripts from plasmid pGFP/A255/CP derivatives, expressing the green fluorescent protein and the MP of *Alfalfa mosaic virus*, which lacks the C-terminal 44 amino acids (AMV 255), *Prunus necrotic ringspot virus* (PNRSV), *Cucumber mosaic virus* (CMV), *Bromovirus* (BMV), *Tobacco mosaic virus* (TMV) or *Cowpea mosaic virus* (CPMV) alone (MP) or fused to the C-terminal 44 amino acids of the MP of AMV (lane 5 in B and 5’ in C) and *Cucumber mosaic virus* (lane 6). M, mock inoculated protoplast. The blots were hybridized with an AMV probe complementary to the 3’ untranslated region. The positions of the Chimeric RNA 3, RNA 4 and additional subgenomic RNA (sgRNA) are indicated in the left margin.

**Fig. 2.** Northern Blot analysis of the accumulation of the *Alfalfa mosaic virus* (AMV) chimeric RNAs in P12 protoplasts. The protoplasts were inoculated with RNA transcripts from constructs assayed in Fig. 1A–MP (A), 1A–MP:A44 (B) and Fig. 4 (C). The chimeric AMV RNAs contains the movement protein gene of AMV (lane 1), *Prunus necrotic ringspot virus* (lane 2), *Cucumber mosaic virus* (lane 3), *Bromovirus* (lane 4), *Tobacco mosaic virus* alone (lane 5 in A) or fused to the C-terminal 44 amino acids of the MP of AMV (lane 5 in B and 5’ in C) and *Cowpea mosaic virus* (lane 6). M, mock inoculated protoplast. The blots were hybridized with an AMV probe complementary to the 3’ untranslated region. The positions of the Chimeric RNA 3, RNA 4 and additional subgenomic RNA (sgRNA) are indicated in the left margin.

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were observed using another virion defective CP mutant (N206; data not shown) (Tenllado and Bol, 2000). These results demonstrate that the virus particles are not required for the cell-to-cell movement mediated by the MP of AMV, BMV, CPMV and TMV. As expected, the MP of TMV, alone or fused to the C-terminal region of the MP of AMV, was able to mediate the transport of the AMV chimeric RNA in absence of virus particles (Figs. 4B and C) supporting the previous results. Interestingly, both BMV:A44 and CPMV:A44 hybrid MPs were able to allow the transport of the AMV chimeric viral RNA in absence of mature virions (Figs. 4D and E). Both BMV and CPMV MPs have been shown to only transport virus particles between cells (Schmitz and Rao, 1996; Wellink and Vankammen, 1989). The results presented clearly show that the MP of both viruses is able to mediate the transport of other viral complexes different than virus particles and (iii) have a common predicted core structure (Melcher, 2000), it is tempting to speculate that the two mechanisms described in the 30K superfamily could represent two variants of the same viral transport system, where the C-terminus of the MP could be adapted to recognize the cognate CP. In addition, the observation that MPs belonging to the 30K superfamily could replace the unrelated MP of viruses having the triple gene block (Ryabov et al., 1998; Solovyev et al., 1996) could point to a common viral transport mechanism.

Material and methods

Plasmids

A modified infectious cDNA 3 clone of AMV that expresses the green fluorescent protein (pGFP/A255/CP) (Sanchez-Navarro and Bol, 2001) was used to exchange the
MP genes. The MP genes of PNRSV, CMV, TMV and CPMV were amplified by PCR from plasmids pPNRSV-PV32 (Aparicio et al., 2003; Herranz and Pallas, 2004), pFny309 (Rizzo and Palukaitis, 1990), pTMV004 (Dawson et al., 1988) and pTM1G (Eggen et al., 1989) with primers listed in Table 1, resulting in fragments with 5' NcoI and 3' NheI sites. These fragments were used to replace the NcoI-NheI fragment of plasmid pGFP/A255/CP (see Fig. 1A). Chimeras with a stop codon at the NheI site expressed PNRSV, CMV, TMV or CPMV MP with wild-type C-termini, whereas chimeras without this stop codon produced MPs with a C-terminal extension corresponding to the C-terminal 44 amino acids of AMV MP (referred as MP:A44 in Fig. 1A).

In the case of the MP of BMV, we used the plasmid pGFP/B1-303/CP (Sanchez-Navarro and Bol, 2001). To extend the C-terminus of the BMV MP protein with the C-terminal 44 amino acid of AMV MP, the NcoI-NheI fragment of the plasmid pB1-303:GFP/CP (Sanchez-Navarro and Bol, 2001) was exchanged with the same fragment of the plasmid pGFP/A255/CP. To introduce the C-terminal 44 amino acids of MP of AMV between amino acids 255 and 256 of the MP of BMV (hybrid BMV255:A44:B48), the hybrid gene BMV255:A44 (Sanchez-Navarro and Bol, 2001) was first subcloned into the pBlue-scripts KS plasmid (Clontech), extended with the sequence of the C-terminal 48 amino acids of the MP of BMV and the resultant MP gene introduced in the construct pGFP/A255/CP by replacing the NcoI-NheI fragment.

The chimera AMV RNA 3 constructs carrying the hybrid MPs BMV:A44, CPMV:A44, TMV:A44 and TMV were modified to express a CP lacking the C-terminal 14 (mutant CP-N206) or 21 amino acids (mutant CP-N199). The introduction of the corresponding mutated CP genes was performed by exchanging the BamHI-PstI fragment obtained from mutants CP-N206 (Tenllado and Bol, 2000) and CP-N199 (van der Vossen et al., 1994).

Glutathione S-transferase (GST) fusion proteins were performed by PCR amplification of the corresponding MP genes using the primers listed in Table 1 and the plasmids described herein as template. The resultant fragments were introduced in the pGEX-KG plasmid (Amersham Pharmacia) using BamHI-HindIII (full-length genes of AMV and PNRSV MPs) or NcoI-HindIII (C-termini of AMV and BMV MPs) restriction enzymes.

**Inoculation of P12 protoplasts and plants**

Plasmids containing wild type or the different mutants of the PNRSV MP were linearized with PstI and transcribed with T7 RNA polymerase. Protoplasts were extracted from transgenic *Nicotiana tabacum* plants that express the polymerase proteins P1 and P2 of AMV (P12 plants; van Dun et al., 1988) and 2.5 × 10^5 protoplasts were inoculated by the polyethylene glycol method (Loesch-Fries et al., 1985) with 6 μl of the transcription mixture. P12 plants were grown and inoculated with RNA transcripts, as described previously (Taschner et al., 1991). GFP expression in plants was analyzed with a Leica TCS SL confocal laser scanning microscope (Leica), with excitation at 488 nm and emission at 510–560 nm.

**GST pull-down assay**

The pull-down assay was performed as described by Stavolone et al. (2005) with some modifications. The challenging GST-fused proteins were produced by overexpression in *E. coli* BL21. Supernatants obtained after bacterial lysis and
centrifugation were incubated with the glutathione-sepharose 4B beads following the manufacturer's instructions. After tree washes in buffer PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4), the GST-fused proteins, already bound to the beads, were incubated for 1 h at 4 °C with a solution of purified AMV virus particles (10 μg/ml in binding buffer: 20 mM Tris–HCl [pH7.5], 100 mM NaCl, 1 mM EDTA). The beads were then washed three times as described by the manufacturer’s instructions using binding buffer. Aliquots (10 μl) of the bound fractions were separated by SDS/PAGE and proteins detected on Western blots with an antiserum against the N terminus of the AMV CP and anti-immunoglobulin G alkaline phosphatase (Promega) as a secondary antibody. Bands were visualized using the NBT/BCIP chromogen (ROCHE) according to the manufacturer’s indications.

Northern blot assays

Total RNA was extracted from protoplasts at 18 h post-inoculation using TRI Reagent (Sigma Steinheim, Germany) as described (Sanchez-Navarro et al., 1997). The RNAs were electrophoresed through formaldehyde-denatured gel and transferred to positively charged nylon membranes (Roche Mannheim, Germany). RNAs were fixed to the membranes by UV cross-linker (700 J/cm2). Hybridization and detection were conducted as previously described (Pallas et al., 1997). The RNAs were transferred to positively charged nylon membranes (Roche Mannheim, Germany). RNAs were fixed to the membranes by UV cross-linker (700 J/cm2). Hybridization and detection were conducted as previously described (Pallas et al., 1997) using a dig-riboprobe (Roche Mannheim, Germany) complementary to the AMV 3’UTR.

Acknowledgments

We thank Professor J. F. Bol and Dr. C. Hernández for the critical reading of the manuscript and V. Navarro for her technical assistance. We thank Professors W.O. Dawson, P. Palukaitis, J. Wellink and J.F. Bol for providing the infectious clones of TMV, CMV RNA 3, CPMV RNA 2 and the AMV RNA 3 derivatives, respectively. We thank Dr. L. Stavolone for her suggestions with pull-down experiments. M. C. Herranz and J. A. Sánchez-Navarro are recipients of a PhD fellowship and a ‘Ramón y Cajal’ contract from the Ministerio de Educación y Ciencia, respectively. This work was supported by Grants BIO 02-4099 and BIO 05-7331 from the Spanish granting agency DGICYT.

References


Table 1

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a Sense primer.

b Antisense primer with a STOP codon before the NheI restriction site.

c Antisense primer in frame with the C-terminal 44 amino acids sequence of the MP of AMV.

d Primers used to fuse MP sequences to the glutathione S-transferase sequence in the pGEX-KG plasmid.


